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Herpes Simplex Virus Gene Expression in Transformed Cells

I. Regulation of the Viral Thymidine Kinase Gene in Transformed L Cells by Products of Superinfecting Virus

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In this paper we show that the expression of the herpes simplex virus type 1 (HSV-1) gene for thymidine kinase (tk) in HSV-transformed cells is subject to regulation by two viral products synthesized during productive infection of these cells with a tk^- mutant of HSV-1. The cell line used in this study is a derivative of tk-deficient mouse L cells that, after exposure to UV-inactivated HSV-1, had acquired the HSV-1 gene for tk (which we term a resident viral gene) and consequently expressed the tk^+ phenotype ($LVtk^+$ cells). Productive infection of these cells with HSV-1(tk^-) at appropriate multiplicities caused significant enhancement of the viral tk activity. The results of several experiments allow us to conclude that this enhancement was due to increased synthesis of tk specified by the HSV-1 gene resident in the $LVtk^+$ cells and that a specific protein made early after infection with HSV-1(tk^-) mediated the enhancement, probably by increasing the production of mRNA from the viral tk gene resident in the $LVtk^+$ cells. Our data also indicate that another HSV-1(tk^-) product acted to turn off tk synthesis. The finding that tk activity continued to increase for a longer time after infection of the $LVtk^+$ cells at 2 PFU/cell than after infection at higher multiplicities suggested the synthesis of a product which inhibited tk synthesis and whose concentration reached critical levels earlier at higher multiplicities of infection. Inhibition of DNA synthesis after infection, a treatment that depresses the synthesis of late viral proteins, prolonged the synthesis of tk in $LVtk^+$ cells infected at either 2 or 5 PFU/cell. Infection of the $LVtk^+$ cells with HSV-2(tk^-) resulted in only small increases in tk activity, indicating some type specificity in recognition of viral products that can modify the expression of the HSV-1 tk gene resident in these cells.

Cells exposed to inactivated herpes simplex virus (HSV) may acquire portions of the viral genome (2, 8, 24, 32), express HSV gene products (1, 3, 4, 6, 7, 31, 33, 34, 37, 38, 47), and exhibit new properties such as those characteristic of malignant transformation (6, 7, 29). The resident viral genetic information is not completely expressed under all conditions (3, 31), however, and transformed cell lines may lose and then subsequently regain the ability to synthesize an HSV gene product, such as thymidine kinase (tk) (3). A problem of central importance in understanding the mechanisms by which viruses transform cells is the definition of factors that regulate expression of the resident viral genes. We have initiated studies along these lines by investigating the conditions that can modify expression of the HSV type 1 (HSV-1) tk gene present in transformed mouse L cells. The results reported here provide evidence that the synthesis of viral tk in these cells is subject to regulation by at least two other HSV-1 gene products—one that enhances and one that inhibits tk synthesis.

Munyon et al. (34) first demonstrated that the HSV-1 or HSV-2 gene for tk could be introduced into mammalian cells and stably transmitted to progeny cells. Specifically, they and others (3, 4, 37) have shown that the treatment of tk-deficient mouse L cells or mouse 3T3 cells with UV-inactivated HSV results in the emergence of tk^+ transformants that can be isolated in the selective HAT medium of Littlefield (27). These tk^+ transformants contain portions of the viral genome (24; Cox and Roizman, personal communication), express several HSV-specific antigens (1), and synthesize tk of viral genetic origin as determined by physical, biochemical, and antigenic analyses (3, 4, 20, 21, 33, 34, 47).

At least two published reports have dealt with the regulation of HSV tk synthesis in cells carrying the viral gene for this enzyme. Lin and Munyon (26) demonstrated that the specific activities of viral tk did not increase coordi-

nately with DNA synthesis in synchronized HSV-transformed mouse L cells, in contrast to the results obtained with the murine tk in normal L cells. They also reported that infection of the tk^+ transformants with tk-deficient HSV resulted in enhancement of tk activity, whereas infection of normal L cells depressed murine tk activity. On the other hand, Davidson et al. (3) found that some, but not all, HSV tk^+ transformants could spontaneously lose the ability to synthesize viral tk although the HSV tk gene was retained. These latter observations suggest the possibility that HSV-specified products might be able to regulate expression of the viral tk gene present in tk^+ transformants.

The objective of the study reported here was, in fact, to determine whether HSV-1 or HSV-2 regulatory gene products could modify the expression of the HSV-1 tk gene that is present in a transformed L cell line isolated by Davidson et al (3). These cells, which we have designated $LVtk^+$, were infected with tk^- mutants of HSV-1 or HSV-2 under conditions such that the particular classes of viral proteins synthesized could be controlled. It was therefore possible to determine whether various input HSV gene products could alter the expression of the resident HSV-1 tk gene that was present in the $LVtk^+$ cells prior to infection with the tk^- mutants.

The design of our experiments was in part based on current information about the regulation of protein synthesis during infection with HSV. Productive infection with either HSV-1 or HSV-2 results in the rapid inhibition of host protein synthesis (10, 35, 43, 45) accompanied by the synthesis of over 50 viral polypeptides, each of which appears to fall into one of at least three coordinately controlled classes (10, 11, 35, 36). For HSV-1, a cascade type of regulation has been described (11, 12, 40) in which a protein of the earliest detectable class (designated α) is required for the synthesis of polypeptides in the next sequential class (designated β) and, similarly, a β protein is required for the synthesis of polypeptides in the third sequential class (designated γ). These regulatory proteins appear to function at the level of transcription and/or processing of mRNA because new transcription in the presence of functional α or β proteins must occur in order for β and γ polypeptides, respectively, to be made (11, 12). Finally a γ function appears to turn off β polypeptide synthesis and a β function turns off α synthesis (11, 12, 40).

For the purposes of our study we made use of the fact that each class of HSV-1 proteins can be defined operationally. Specifically, α polypeptides can be differentiated from those of the other two classes by the criterion that only α polypeptides are made in infected cells treated with an inhibitor of protein synthesis during the first few hours postinfection and then with an inhibitor of RNA synthesis at the time that protein synthesis is restored; apparently only α mRNA's can be produced during the interval prior to the total block in transcription when all new viral protein synthesis is inhibited (11, 23, 40). Polypeptides of the β and γ classes can be differentiated from each other on the basis of their rates of synthesis in infected cells treated with inhibitors of DNA synthesis (11, 36). The synthesis of γ polypeptides is depressed, although not necessarily abolished, in the absence of viral DNA synthesis, whereas the synthesis of β polypeptides, of which tk is an example (9), is unaffected or may be enhanced, probably because a γ function is normally responsible for the shutoff of β polypeptide synthesis (11, 12).

Because the virus mutants used in this study were defective in the production of tk and could otherwise replicate normally (5, 17, 18, 46, 48), infection of the $LVtk^+$ cells under the various conditions described above allowed us to assess the effects of both α and γ input products on synthesis of enzyme from the HSV-1 tk gene present in the $LVtk^+$ cells.

(A preliminary account of this work was presented at the VIIth International Symposium on Comparative Research on Leukemia and Related Diseases held in Copenhagen on 13 to 17 October 1975.)

MATERIALS AND METHODS

Chemicals. Puromycin dihydrochloride was obtained from Sigma Chemical Co. (St. Louis, Mo.), cycloheximide and cytosine arabinoside from Nutritional Biochemical Corp. (Cleveland, Ohio), and actinomycin D from Calbiochem (La Jolla, Calif.). [2-^{14}C]thymidine (50 mCi/mmol), [*methyl*-^{3}H]thymidine (40 to 60 Ci/mmol), [5-^{3}H]uridine (25 Ci/mmol), and L-[^{35}S]methionine (100 to 400 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). Phosphonoacetic acid was a gift from L. R. Overby, Abbott Laboratories, Chicago, Ill.

Cell lines. Three different cell lines were used in the studies described in this report: mouse L929 cells (L cells) obtained from J. M. Moulder (University of Chicago), a tk-deficient line of mouse L cells (Ltk^- cells) isolated by Kit et al. (19), and a derivative of the Ltk^- cell line that carries the HSV-1 tk gene ($LVtk^+$ cells) and was isolated by Davidson et al. (3) after infection of the Ltk^- cells with UV-irradiated HSV-1 (strain VR3). Both the Ltk^- cells and $LVtk^+$ cells were obtained from R. Davidson (Harvard Medical School, Boston, Mass.). Preliminary experiments (B. Cox and B. Roizman, personal communication) indicate that approximately 30% of the HSV-

1 genome is present in the particular LVtk$^+$ cell line used in this study. The L cells and Ltk$^-$ cells were passaged in Ham F10 medium supplemented with 10% inactivated fetal calf serum, penicillin (150 U/ml), streptomycin (150 μg/ml), and tylocine (60 μg/ml), whereas the LVtk$^+$ cells were maintained in Ham F10 medium supplemented with 10% inactivated fetal calf serum, penicillin, streptomycin, tylocine, 10^{-5} M hypoxanthine, 1.6×10^{-5} M thymidine, and 4.4×10^{-7} M methotrexate—a modification of the HAT medium of Littlefield (27) that was designed for the selection of cells expressing the tk$^+$ phenotype. The Ltk$^-$ cells and LVtk$^+$ cells were used for experiments on day 8 after transfer or 3 days after reaching confluence.

Viruses. A tk$^-$ mutant of HSV-1 (B2006) and its tk$^+$ parental strain (C1 101) were obtained from S. Kit (Baylor University, Houston, Tex.). The properties of these viruses were described by Kit and Dubbs (5, 17, 18). A tk$^-$ mutant derived from the BRY strain of HSV-2 (46, 48) was provided by M. Thouless (University of Birmingham, Birmingham, England). Stocks of each of these virus isolates were prepared as previously described (42) from HEp-2 cells that had been infected at a low multiplicity (0.01 PFU/cell); the stocks were titered on HEp-2 cell monolayers (41).

Infection and extraction of cells. Confluent monolayer cultures containing 3×10^7 or 5×10^6 cells were infected at various input multiplicities of virus as indicated below. The cultures were exposed to virus in a small volume of phosphate-buffered saline (PBS) supplemented with 1% calf serum and glucose, by continuous shaking for 2 h at 37°C. After replacing the virus inoculum with Ham F-10 medium, incubation of the cultures was continued at 37°C. Mock-infected cultures were treated exactly as described for infected cultures except that virus was omitted; the mock inoculum contained an uninfected HEp-2 cell extract prepared in the same fashion as the virus stocks. At various times after exposure to virus or to a mock inoculum, the cells were scraped from the glass surface, collected by centrifugation, washed with cold PBS, and frozen at −70°C in a small volume of PBS. Within 24 h the cells were thawed and homogenized by freeze-thawing or by sonic treatment on ice for 1 min. The cell lysates were then tested for tk activity without further treatment. Lysates prepared from replicate cultures at various times after infection did not differ significantly in protein concentration, as determined by the Lowry assay (28), indicating that infection did not alter the total protein mass of the cultures and that recovery of protein during the harvest and processing of the cells was constant. Lysates prepared from replicate mock-infected cultures showed a slight increase in protein concentration with time during the course of most experiments.

tk assay. Portions (150 μl) of cell lysates containing either 6.7 or 3.7 mg of protein per ml were mixed with 100 μl of a solution containing 0.25 M phosphate buffer, pH 7.4, 12.5 mM ATP, 0.125 M $MgCl_2$, and 2.5 μCi of [^{14}C]thymidine (nominally 50 mCi/mmol). The reaction mixtures were incubated for 15 min in a 38°C water bath and then boiled for 2.5 min to stop the reaction. Following a modification of the procedure reported by Jamieson and Subak-Sharpe (16), 50-μl aliquots of each mixture were spotted in duplicate on squares of Whatman DEAE paper, which were then washed twice with 1 mM ammonium formate, once with distilled water, and once with ethanol. The DEAE paper squares were dried and immersed in a toluene-based scintillation fluid for quantitation of the adsorbed [^{14}C]TMP. Enzyme activity is expressed as counts per minute of [^{14}C]TMP formed (the average value of the duplicate determinations) per 10^6 cells in the culture from which the lysate was obtained. The activity was proportional to the amount of lysate added to the reaction mixture over the range of protein concentrations used.

Neutralization of tk activity. An anti-HSV-1 serum that had neutralizing activity against the viral tk was a gift from M. Terni (University of Bologna, Bologna, Italy); this antiserum (designated Ra41) was obtained from a rabbit that had received multiple injections of HEp-2 cells infected with HSV-1. Nonimmune rabbit serum was obtained from an untreated animal. Neutralization of tk activity was performed by mixing appropriate volumes of antiserum with 50 μl of cell lysate. All mixtures were brought to a final volume of 150 μl with cold PBS and, after 3 h at 0°C, were assayed for tk activity as described above. The results shown in Fig. 1 demonstrate that the anti-HSV-1 serum effectively neutralized the tk activity induced after HSV-1(tk$^+$) infection of Ltk$^-$ cells and also neutralized the activity present in LVtk$^+$ cells, whereas it had little or no specific activity against the murine tk of uninfected L cells. The 25 to 30% reduction in murine tk activity was apparently a nonspecific effect of adding serum to the reaction mixture because serum from unimmunized rabbits caused a similar reduction. Nonimmune serum also had a slightly inhibitory effect on the HSV-1 tk activity. The residual activity detected after the neutralization of the viral enzyme with 100 μl of antiserum was probably due primarily to a mitochondrial activity that has been described in the Ltk$^-$ cells (22); the low level of tk activity detectable in uninfected Ltk$^-$ cells was not neutralizable by the anti-HSV-1 serum (data not shown).

Radiolabeling of cells. To ascertain the effectiveness of various inhibitors of macromolecular synthesis used in this study, replicate cultures of LVtk$^+$ cells infected with HSV-1(tk$^-$) at 2 PFU/cell were incubated with inhibitor under the conditions described below or were maintained as untreated infected controls and were then pulse labeled for a 30-min interval by incubation with [^{35}S]methionine (5 μCi/ml in methionine-deficient medium 199 supplemented with 1% fetal calf serum), [^{3}H]uridine (10 μCi/ml in medium 199-1% fetal calf serum), or [^{3}H]thymidine (10 μCi/ml in medium 199-1% fetal calf serum). The radioactive medium for the treated cultures contained the appropriate inhibitor. Immediately after the labeling period, the cells were washed with PBS, scraped from the glass surface, collected by centrifugation, suspended in a small volume of PBS, and sonically treated. Portions of the cell lysates were then precipitated with trichlo-

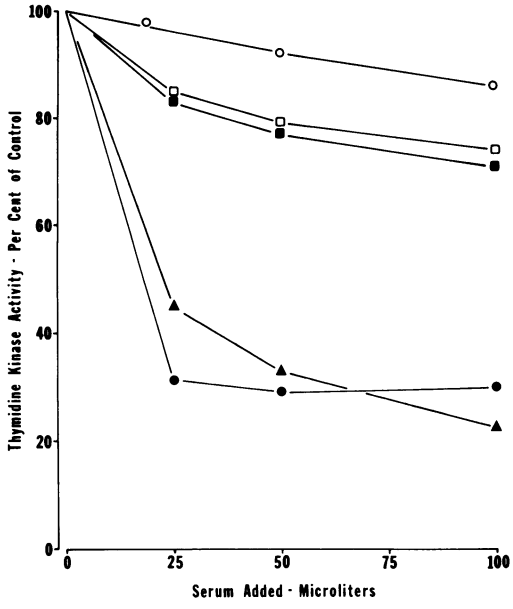

FIG. 1. *Antibody neutralization of viral tk activity present in extracts prepared from uninfected LVtk⁺ cells or from Ltk⁻ cells infected with HSV-1(tk⁺). An extract from normal L cells was also tested in the neutralization assay as a control. The indicated volumes of anti-HSV-1 serum (filled symbols) or of nonimmune rabbit serum (open symbols) were mixed with portions of cell extract as described in the text, and the residual tk activity was then determined. Symbols: ●, ○, extract from uninfected LVtk⁺ cells, with tk activity of 10,600 cpm/10⁶ cells in the absence of serum; ▲, extract prepared at 7 h postinfection from Ltk⁻ cells infected with 10 PFU of HSV-1(tk⁺) per cell, with tk activity of 22,500 cpm/10⁶ cells in the absence of serum; ■, □, extract from uninfected L cells, with tk activity of 10,800 cpm/10⁶ cells in the absence of serum.*

roacetic acid for quantitation by liquid scintillation counting of radioactivity incorporated into macromolecules.

RESULTS

Enhancement of tk activity in LVtk⁺ cells by infection with HSV-1(tk⁻). Infection of the LVtk⁺ cells with HSV-1(tk⁻) significantly enhanced their tk activity (Fig. 2). The kinetics and levels of activity observed, however, were highly dependent on the multiplicity of infection. Specifically, tk activity in cells infected with 1 and 2 PFU/cell increased at about the same rate, but the enhancement was detected a few hours earlier in cells infected at the higher multiplicity. A comparison of cells infected with 2 and 5 PFU/cell reveals that tk activity increased at about the same time and rate, but at the higher multiplicity the activity reached a plateau earlier and at a lower level. The levels of tk activity detected after infection with 10 PFU/cell did not differ from those present in mock-infected control cultures, a result that is somewhat inconsistent with the data of Lin and Munyon (26) who reported enhancement after infection at this multiplicity. Their results are not readily comparable with ours, however, because different LVtk⁺ cell lines were used and also, in their study, infection at multiplicities other than 10 PFU/cell was not done.

Mock infection sometimes resulted in the gradual elevation of tk activity, but this elevation differed both qualitatively and quantitatively from that observed after infection with appropriate multiplicities of virus and was probably due to the addition of fresh medium and/or uninfected cell extract to resting monolayers. It should be emphasized that the enhanced tk activity detected after infection of the LVtk⁺ cells was not due to enzyme specified by the input viral genome because, as demonstrated by Dubbs and Kit (5, 17) and as shown in Fig. 2, the virus used in these experiments

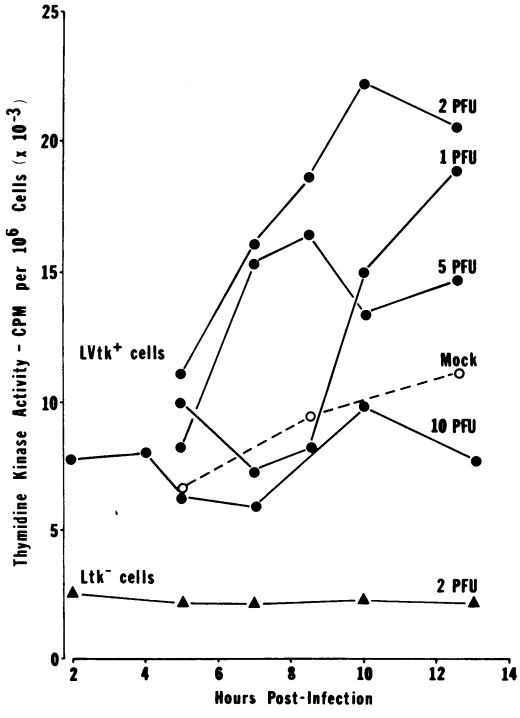

FIG. 2. *Enhancement of tk activity in the LVtk⁺ cells after infection with HSV-1(tk⁻). tk assays were done on extracts from LVtk⁺ cells (●) or from Ltk⁻ cells (▲) at the indicated times after infection with HSV-1(tk⁻) at the input multiplicities shown or after mock infection of the LVtk⁺ cells (○).*

was defective in the production of active tk. Moreover, this mutant fails to synthesize the tk polypeptide according to electrophoretic (44) and antigenic analyses (13). It should also be noted that the HSV-1(tk⁻)-induced enhancement of tk activity in the LVtk⁺ cells is in marked contrast to the inhibitory effects of HSV infection on the synthesis of cellular proteins (10, 35, 43, 45). We confirmed previous reports (5, 26) that infection of L cells with HSV-1(tk⁻) depressed murine tk activity and also demonstrated that infection of the LVtk⁺ cells with HSV-1(tk⁻) resulted in inhibition of cellular protein synthesis (data not shown).

To investigate the specificity of the HSV-1(tk⁻)-induced enhancement in tk activity, the LVtk⁺ cells were also infected with HSV-2(tk⁻), a virus that is genetically related to but serologically differentiable from HSV-1. From the results presented in Fig. 3, it is evident that infection with HSV-2(tk⁻) did not cause significant changes in the levels of tk activity whether the virus was added at multiplicities of 1, 2, or 5 PFU/cell. Because both viruses were capable of replicating in the LVtk⁺ cells (Table 1), we conclude that the enhancement of tk activity observed after HSV-1(tk⁻) infection (Fig. 2) required a specific set of functions supplied by HSV-1 and was not merely a consequence of physiological changes accompanying HSV replication.

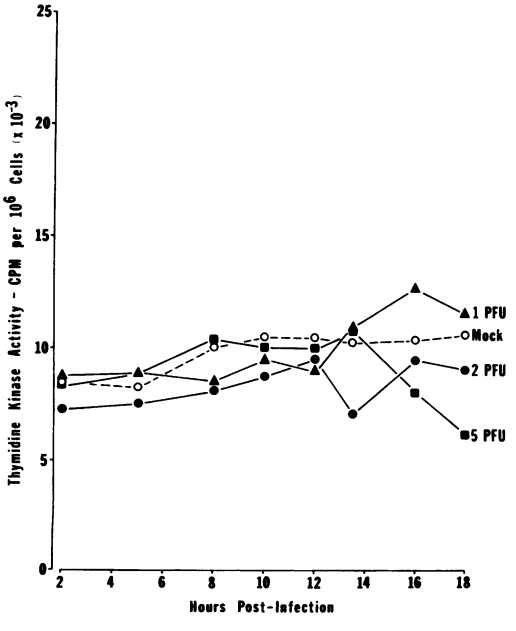

FIG. 3. *tk activity in extracts from LVtk⁺ cells at the indicated times after infection with HSV-2(tk⁻) at the input multiplicities shown (▲, ●, ■) or after mock infection (○).*

TABLE 1. *Replication of HSV-1(tk⁻) and HSV-2(tk⁻) in LVtk⁺ cells*

Virus	PFU/culture (×10⁻⁵)[a]		
	2 h	18 h	21 h
HSV-1(tk⁻)	6.8	730	680
HSV-2(tk⁻)	9.9	245	250

[a] Replicate cultures of LVtk⁺ cells (4 × 10⁶ cells/culture) were infected with HSV-1(tk⁻) or HSV-2(tk⁻) at an input multiplicity of 2 PFU/cell. At 2, 18, and 21 h postinfection, the cells were scraped, collected by centrifugation, sonically treated in 1 ml of medium plus skimmed milk, and frozen at −70°C. Infectivity titrations were performed on Vero cell monolayers under an agarose overlay.

Antigenic properties of the tk synthesized after infection of the LVtk⁺ cells. As described above, the hyperimmune rabbit serum prepared against HSV-1(tk⁺)-infected HEp-2 cells effectively neutralized the HSV-1 tk activity present in extracts from LVtk⁺ cells or from Ltk⁻ cells infected with HSV-1(tk⁺), but had little, if any, specific activity against the murine tk of normal L cells (Fig. 1). Table 2 shows that the enzyme detected in LVtk⁺ cells infected with HSV-1(tk⁻) also had antigenic properties of the HSV-1 tk.

Evidence that virus-induced enhancement of tk activity in LVtk⁺ cells required new protein synthesis after infection. Experiments were carried out to determine whether the enhancement of tk activity observed after HSV-1(tk⁻) infection required new protein synthesis, both during the time of maximal increase in enzyme activity and prior to detectable increases in activity. In the first instance, we wished to provide evidence that new tk synthesis accounted for the increase in enzyme activity. In the second, we wished to determine whether a protein made early in infection played a critical role in the enhancement of tk synthesis.

To determine whether inhibition of protein synthesis could arrest the increase in tk activity, replicate cultures of LVtk⁺ cells were infected with HSV-1(tk⁻) at 2 PFU/cell and puromycin was added to some cultures at 7 h postinfection, by which time increases in tk activity were already evident. The concentration of drug used (50 μg/ml) was sufficient to reduce protein synthesis to 3.9% of the control levels, as determined by measuring the incorporation of [³⁵S]methionine into proteins of drug-treated and untreated infected LVtk⁺ cells. Figure 4 shows that the addition of puromycin immedi-

TABLE 2. *Neutralization of tk activity by anti-HSV-1 serum*

Extract	tk activity (cpm/10^6 cells)		
	No antiserum	100 μl of antiserum	Neutralization (%)
LVtk$^+$ cells infected with HSV-1(tk$^-$)[a]	18,000	4,600	74
Uninfected LVtk$^+$ cells	10,600	3,200	70
Ltk$^-$ cells infected with HSV-1(tk$^+$)[b]	22,500	5,000	78
Uninfected L cells	10,800	7,700	29

[a] Extracts were prepared at 10 h after infection with HSV-1(tk$^-$) at 2 PFU/cell.

[b] Extracts were prepared at 7 h after infection with HSV-1(tk$^+$) at 10 PFU/cell.

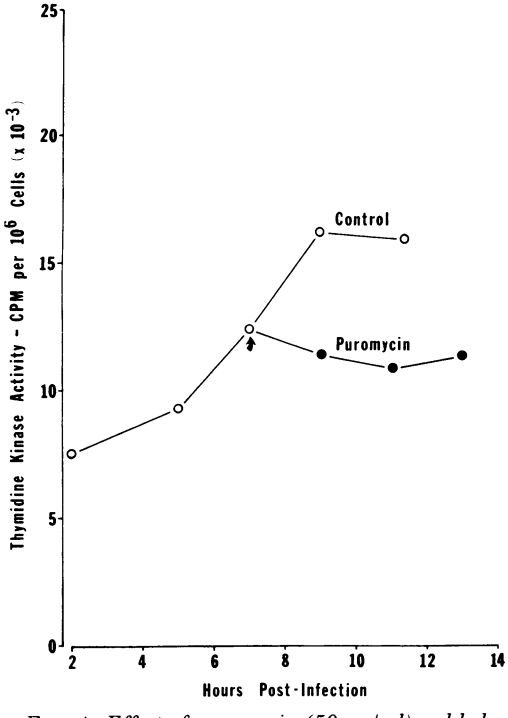

FIG. 4. *Effect of puromycin (50 μg/ml), added at 7 h postinfection, on the enhancement of tk activity resulting from infection of the LVtk$^+$ cells with HSV-1(tk$^-$) at 2 PFU/cell. Symbols: ●, extracts from puromycin-treated infected cells; ○, extracts from infected cells that did not receive the drug.*

ately interrupted the increase in tk activity that continued in replicate cultures not treated with the drug. These results are consistent with the hypothesis that de novo synthesis of enzyme accounts for the increased tk activity detected after infection, particularly in light of reports that the HSV-1 tk is composed of a single species of polypeptide (13) and that no other viral or cellular polypeptide can be detected in association with the active enzyme (14).

To investigate the possibility that a protein made early after infection might play a role in inducing the enhanced levels of tk, replicate cultures were infected with 2 PFU of HSV-1(tk$^-$) per cell in the absence or in the presence of cycloheximide at a concentration (50 μg/ml) sufficient to reduce protein synthesis to 1.5% of the control levels. Upon removal of the cycloheximide at 5 h postinfection, the rate of protein synthesis recovered to control levels within 30 min (data not shown), but increases in tk activity could not be detected until about 5 h later (Fig. 5). The levels of tk activity attained in the cycloheximide-treated cultures were comparable to those observed in the untreated infected cultures but only after a significant delay. These results suggest that the enhancement of tk activity required the function of a protein normally made during the first 5 h postinfection.

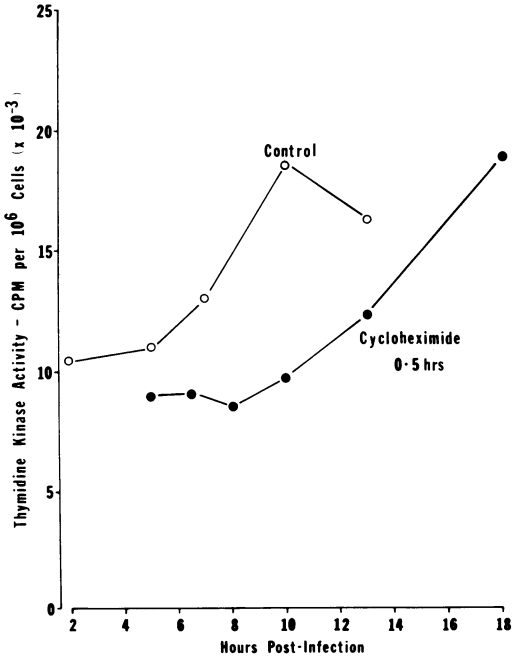

FIG. 5. *Delay of the HSV-1(tk$^-$)-induced enhancement in tk activity by treatment of the infected LVtk$^+$ cells with cycloheximide (50 μg/ml) during the first few hours of infection. Replicate cultures of LVtk$^+$ cells were infected with HSV-1(tk$^-$) at 2 PFU/cell in the presence (●) or absence (○) of cycloheximide. The inhibitor was washed out of the treated cultures at 5 h postinfection.*

Prevention of enhanced tk synthesis by sequential inhibition of protein synthesis and RNA synthesis after infection. The synthesis of viral tk in cells infected with HSV-1(tk$^+$) requires the prior synthesis of an α protein (9, 11), whose function is apparently to ensure the proper transcription and processing of the β mRNA's (11, 12, 23, 40), including tk mRNA. Viral tk cannot be synthesized in HSV-1(tk$^+$)-infected cells that are treated with cycloheximide during the first few hours of infection (to allow accumulation of α mRNA's) and then treated with actinomycin D at the time cycloheximide is removed (9), presumably because the synthesis of functional β mRNA's requires that transcription take place in the presence of an α protein.

To determine whether enhancement of tk synthesis in the infected LVtk$^+$ cells could also be prevented by the sequential inhibition of protein and RNA synthesis, replicate cultures were infected with HSV-1(tk$^-$) at 2 PFU/cell and incubated with cycloheximide (50 μg/ml) from 0 to 4 h postinfection. At the time the cycloheximide was removed, actinomycin D was added at a concentration (10 μg/ml) sufficient to reduce [^{3}H]uridine incorporation to 1.5% of the control levels. Other sets of cultures were also infected and were either treated with cycloheximide alone or were untreated. Figure 6 shows that sequential treatment with cycloheximide and actinomycin D prevented the enhancement of tk activity, whereas the treatment with cycloheximide alone simply delayed the synthesis of enzyme as was already shown in Fig. 5. These results, coupled with the data presented in the preceding section, demonstrate that the enhanced synthesis of tk in the infected LVtk$^+$ cells required new transcription in addition to the synthesis of an early viral protein.

Enhancement of tk activity is accentuated by inhibition of DNA synthesis after infection. The finding that the enhancement in tk activity was maximal after infection of the LVtk$^+$ cells with HSV-1(tk$^-$) at 2 PFU/cell, but was less pronounced or undetectable at higher multiplicities (Fig. 2), raised the possibility that a γ viral product, synthesized in larger amounts at the higher multiplicities of infection, might act to turn off viral tk synthesis, thus antagonizing the effects of the inducing function. One prediction of this hypothesis is that interference with the synthesis of γ products should prevent or delay the shutoff of tk synthesis in the infected LVtk$^+$ cells and should allow the accumulation of higher enzyme levels. To test this prediction, replicate cultures of LVtk$^+$ cells were infected with HSV-1(tk$^-$) at 2

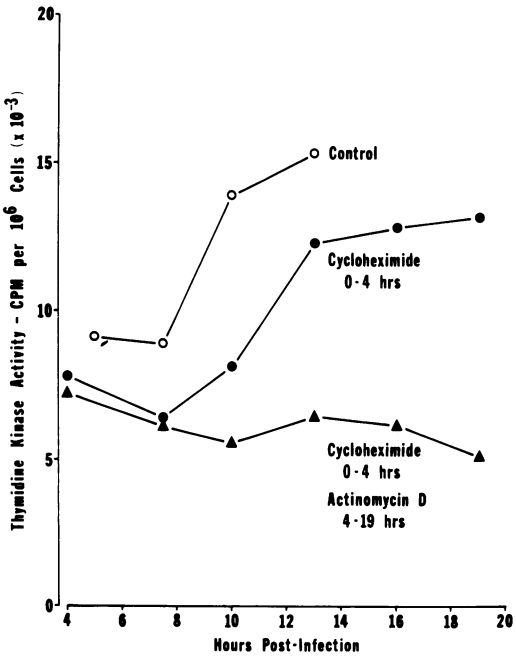

FIG. 6. *Prevention of the HSV-1(tk$^-$)-induced enhancement in tk activity by sequential treatment of the infected LVtk$^+$ cells with cycloheximide and actinomycin D. Three sets of replicate cultures were infected with HSV-1(tk$^-$) at 2 PFU/cell. One set received no drugs (○), whereas the other two were treated with cycloheximide (50 μg/ml) alone (●) or with cycloheximide and then actinomycin D (10 μg/ml) (▲) for the intervals of time indicated.*

or 5 PFU/cell in the absence or in the presence of cytosine arabinoside at a concentration (50 μg/ml) sufficient to reduce [^{3}H]thymidine incorporation into DNA to 0.3% of the control levels; inhibition of HSV DNA synthesis after infection is known to drastically reduce, although it may not abolish, the synthesis of γ proteins, whereas the synthesis of α and β proteins is either unaffected or enhanced (11, 36). Figure 7 shows that inhibition of DNA synthesis after infection at either multiplicity resulted in significantly greater enhancement of tk activity as compared with the untreated infected controls. Incubation of the infected LVtk$^+$ cells in the presence of cytosine arabinoside did not completely abolish the high multiplicity effect, however, in that the enhancement of tk activity was still greater at 2 PFU/cell than at 5 PFU/cell. This result is not unexpected, assuming that the putative γ inhibitory protein could still be made, albeit in reduced quantities, in the absence of new viral DNA synthesis because the amount of inhibitor made would depend on the number of parental viral genomes per cell (39; unpublished data).

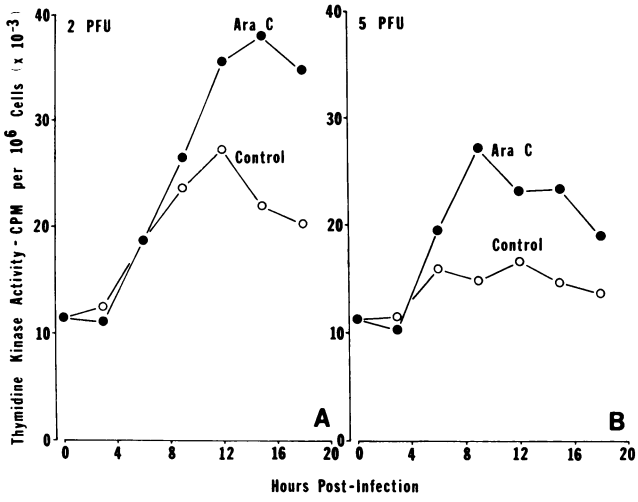

FIG. 7. *Effect of a DNA synthesis inhibitor on the enhancement of tk activity observed after infection of the LVtk⁺ cells with HSV-1(tk⁻). Replicate cultures were infected with HSV-1(tk⁻) at either 2 PFU/cell (A) or 5 PFU/cell (B) and were incubated in the absence (○) or in the presence (●) of cytosine arabinoside (50 μg/ml) added at the time of infection.*

A comparison of the kinetics of HSV-1(tk⁻)-induced tk enhancement in the presence and absence of cytosine arabinoside after infection (Fig. 7) suggests that the initial stages of the enhancement process were unaffected by the inhibition of DNA synthesis. The increase in tk activity continued at a linear rate for a longer time, however, in the presence of cytosine arabinoside than in its absence, consistent with the hypothesis that the shutoff of tk synthesis could be delayed by interference with the synthesis of γ products. We found that the use of phosphonoacetic acid, reported to be a specific inhibitor of herpesvirus DNA synthesis (15, 25, 30), instead of cytosine arabinoside gave similar results to those presented in Fig. 7. Specifically, a 4.5-fold enhancement of tk activity was observed after infection of LVtk⁺ cells with HSV-1(tk⁻) at 2 PFU/cell in the presence of phosphonoacetic acid (50 μg/ml), whereas without inhibitor only a 3-fold enhancement was evident. The treatment of the LVtk⁺ cells with phosphonoacetic acid alone or with cytosine arabinoside alone, in the absence of HSV-1(tk⁻) infection, did not cause an enhancement in viral tk activity, indicating that the increase in enzyme activity observed in the presence of these drugs was a specific consequence of virus infection.

Because of our finding that the inhibition of DNA synthesis accentuated the enhancement of tk synthesis observed after infection with HSV-1(tk⁻) (Fig. 7), we tested the possibility that infection of the LVtk⁺ cells with HSV-2(tk⁻) in the presence of cytosine arabinoside might result in a detectable elevation in tk activity. Figure 8 shows that higher levels of tk activity were detected in the cytosine arabinoside-treated cultures than in the untreated infected controls, but the levels of enzyme activity observed did not exceed even twice that of the uninfected cells. The differential effect of HSV-1(tk⁻) and HSV-2(tk⁻) on synthesis of HSV-1 tk by the LVtk⁺ cells may be due in part to the fact that the time scale of HSV-2 protein synthesis is more compressed than that of HSV-1 protein synthesis (35), leading to the earlier accumulation of late viral products, but a comparison of the results presented in Fig. 7 and 8 suggests that there may be a degree of type specificity in the induction phase of the enhancement phenomenon.

DISCUSSION

The major conclusion of this study is that synthesis of tk specified by the resident HSV-1 gene in LVtk⁺ cells is subject to regulation by products of a superinfecting HSV-1 genome. We identified two regulatory phenomena that occurred after infection of the LVtk⁺ cells with HSV-1(tk⁻). The first was a significant increase in viral tk activity, initiated between 4 and 6 h after the addition of the virus at 2 or 5 PFU/cell (Fig. 2). The second and later effect was a specific arrest of the increase in tk activity; this arrest occurred earlier at the higher multiplicities (Fig. 2) and could be partially prevented by the inhibition of DNA synthesis (Fig. 7). In this discussion we shall be concerned with the source of genetic information

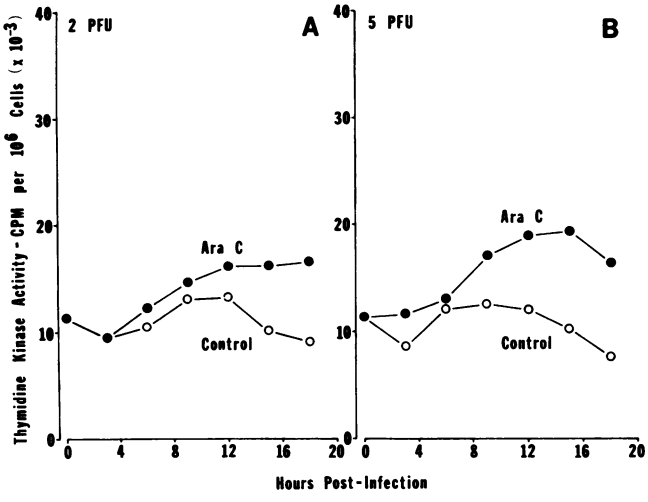

FIG. 8. *Effect of a DNA synthesis inhibitor on the levels of tk activity observed after infection of the LVtk⁺ cells with HSV-2(tk⁻). Replicate cultures were infected with HSV-2(tk⁻) at either 2 PFU/cell (A) or 5 PFU/cell (B) and were incubated in the absence* (○) *or in the presence* (●) *of cytosine arabinoside (50 μg/ml) added at the time of infection.*

for the tk synthesized after infection and with the functions responsible for the enhancement and inhibition of tk synthesis.

Source of genetic information for the tk activity expressed in infected LVtk⁺ cells. Davidson et al. (3) concluded on the basis of electrophoretic analyses that the transformed cell line used in this study expressed the HSV-1 tk activity. Our antibody neutralization data, which are summarized in Fig. 1, confirm this conclusion. Table 2 shows that the new tk activity detected after the infection of the LVtk⁺ cells with HSV-1(tk⁻) was also HSV specific, and we conclude that it was a product of the HSV-1 tk gene resident in the LVtk⁺ cells because the HSV-1(tk⁻) virus is unable to specify an active enzyme (Fig. 2; references 5 and 17) and even fails to make an inactive tk polypeptide (44). Finally, the elevation in tk activity observed after the infection appears to be the result of new enzyme synthesis rather than activation of preexisting molecules because an inhibitor of protein synthesis immediately blocked further increases in tk activity after enhancement was already evident (Fig. 4). It should be pointed out that the HSV-1 tk is probably composed of a single polypeptide (13), and there is no evidence that any other viral or cellular polypeptide can modulate its activity (14).

Enhancement of tk activity in LVtk⁺ cells infected with HSV-1(tk⁻). Our data rule out the possibility that the HSV-1(tk⁻)-induced enhancement in tk synthesis resulted from the elimination of a repressor whose synthesis was inhibited by virus infection. In the first place, infection of the LVtk⁺ cells with HSV-2(tk⁻) or with high multiplicities of HSV-1(tk⁻) was relatively ineffective at enhancing tk activity (Fig. 2 and 3), even though inhibition of host protein synthesis would occur under these conditions as well as after infection with HSV-1(tk⁻) at 2 PFU/cell. In addition, we found that the treatment of the LVtk⁺ cells with cycloheximide alone, in the absence of HSV-1(tk⁻) infection, did not enhance tk activity (data not shown). Our interpretation of the data presented in this report is that a protein specified by the input HSV-1(tk⁻) genome enhanced the synthesis of viral tk specified by the HSV-1 gene resident in LVtk⁺ cells and that this putative regulatory protein probably acted at the level of transcription or post-transcriptional processing of mRNA and belongs to the α class of HSV-1 proteins.

The evidence that supports this hypothesis is as follows. (i) The striking increase in tk activity observed after infection of the LVtk⁺ cells with HSV-1(tk⁻) (Fig. 2 and 7), contrasted with the small and perhaps insignificant changes seen after HSV-2(tk⁻) infection (Fig. 3 and 8), suggests that a specific HSV-1(tk⁻) product, rather than physiological changes accompanying virus replication, was responsible for the enhancement. (ii) The HSV-1(tk⁻)-induced increase in tk activity was delayed by the inhibition of protein synthesis during the first few hours of infection (Fig. 5 and 6), suggesting that a protein made early in infection played a critical role in the enhancement. (iii) The HSV-

1(tk⁻)-induced enhancement in tk synthesis was prevented by sequential treatment of the infected cells with cycloheximide and then with actinomycin D (Fig. 6), a protocol that allows α proteins to be made but that prevents the production of mRNA's for β and γ proteins because only α mRNA's can be made during the inhibition of protein synthesis prior to the total block of transcription by actinomycin D (11, 23, 40). The finding that the enhancement of tk synthesis under these conditions required new transcription could reflect the need for the synthesis of β or γ proteins specified by the input viral genome. The experiment shown in Fig. 7, however, rules out the possibility that a γ protein is required for the enhancement because the depression of γ protein synthesis by inhibition of DNA synthesis actually potentiated the increase in tk activity. Inasmuch as the synthesis of tk from the genome of HSV-1(tk⁺) is apparently induced by an α function and turned off by a γ function (9), it seems most likely that the specific HSV-1 protein required for enhancement of tk synthesis in the LVtk⁺ cells is an α protein and that the new transcription required is the α-regulated production of mRNA from the cell's resident HSV-1 tk gene.

Inhibition of tk synthesis in LVtk⁺ cells infected with HSV-1(tk⁻). Our data indicate that another product specified by the input HSV-1(tk⁻) genome acted to turn off tk synthesis in the LVtk⁺ cells and that this second regulatory product may belong to the γ class of HSV-1 proteins. The finding that tk activity ceased to increase earlier in the LVtk⁺ cells infected at the higher multiplicities (Fig. 2) is consistent with the synthesis of a late viral inhibitory product whose concentration reaches a critical level earlier at the higher multiplicities. High-multiplicity infection tends to compress the viral replicative cycle, causing the earlier appearance of all viral proteins, particularly those in the β and γ classes, as might be expected (39; unpublished data). Our hypothesis that a γ gene product of the input HSV-1(tk⁻) was responsible for the turnoff of tk synthesis is supported by the data shown in Fig. 7. Inhibition of viral DNA synthesis, which is known to depress the synthesis of γ proteins (11, 36), had the effect of prolonging tk synthesis in the LVtk⁺ cells infected with HSV-1(tk⁻). It should be noted that the inhibition of DNA synthesis in HSV-1(tk⁺)-infected cells also prolongs the synthesis of viral tk (9).

Implications and significance of the data. There are two immediate implications of the findings reported here. The first is that, although the environment of the HSV-1 tk gene is probably considerably different in the LVtk⁺ cells than in the viral genome, the recognition signals that allow HSV-1-specified regulatory products to enhance or inhibit expression of the gene must be intact in the transformed cell. Second, the possibility exists that products of HSV-1 regulatory genes could participate in regulating viral tk expression in the untreated LVtk⁺ cells, provided the appropriate genes were incorporated during the tk⁺ transformation. Assuming that an HSV-1 α protein were required for expression of the viral tk in the LVtk⁺ cells, then tk⁺ transformation would depend on the stable incorporation of at least two viral genes into the cells. If the HSV-1 α protein is actually expressed in the LVtk⁺ cell line used in this study, its availability must be limited or intermittent because, otherwise, the viral tk would always be synthesized at high levels and it probably would not be possible to enhance the synthesis by infection with HSV-1(tk⁻). With regard to the possibility that the γ regulatory gene might also become stably incorporated into the LVtk⁺ cells, it is of interest that some of the transformants isolated by Davidson et al. (3), including the one used in this study, can spontaneously lose the ability to synthesize viral tk although the HSV-1 tk gene is retained. These phenotypic revertants can be isolated by growth and cloning of the cells in nonselective medium, and preliminary studies (Buttyan and Spear, manuscript in preparation) indicate that such cells cannot be induced to synthesize tk by infection with HSV-1(tk⁻). The most important observation, however, is that these cells, unlike the phenotypically tk⁺ cells used in this study, will not support virus replication. The possibility exists, therefore, that a product capable of inhibiting viral functions, and perhaps of viral origin, may be expressed under certain conditions in some of the LVtk⁺ cell lines.

The studies reported here have implications for the induction and maintenance of biochemical or oncogenic transformation by HSV because of our findings that the expression of cell-associated viral genes can be influenced by the action of virus-specified regulatory functions. Thus, the frequency of a detectable transformation event may depend not only on the incorporation of a viral gene whose product alters the phenotype of the cell, but also on the presence or absence of HSV genes that could regulate the synthesis of this gene product. The possibility also exists that virus-specified regulatory proteins could play a more direct role in oncogenic transformation.

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